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**14. ABSTRACT**

Inflammation of the prostate is a risk factor for the development of prostate cancer. In the aging prostate, regions of inflammatory atrophy are foci for prostate epithelial cell transformation. Expression of the suppressor protein NKX3.1 is reduced in regions of inflammatory atrophy and in preinvasive prostate cancer. Inflammatory cytokines tumor necrosis factor (TNF)-alpha and interleukin-1B accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal degradation. The effect of TNF-alpha is mediated via the C-terminal domain of NKX3.1 where phosphorylation of serine 196 is critical for cytokine-induced degradation. Mutation of serine 196 to alanine abrogates phosphorylation at that site and the effect of TNF-alpha on NKX3.1 ubiquitination and protein loss. This is in contrast to control of steady-state NKX3.1 turnover, which is mediated by serine 185. Mutation of serine 185 to alanine increases NKX3.1 protein stability by inhibiting ubiquitination and doubling the protein half-life. A third C-terminal serine at position 195 has a modulating effect on both steady-state protein turnover and on ubiquitination induced by TNF-alpha. Cellular levels of the NKX3.1 tumor suppressor are affected by inflammatory cytokines that target C-terminal serine residues to activate ubiquitination and protein degradation. We suggest that the inhibition of inflammation or effector kinases may be useful approaches to prostate cancer prevention.

**15. SUBJECT TERMS**
Tumor Suppressor, NKX3.1, Cytokines, Inflammation

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## Introduction

One of the earliest events in prostate cellular transformation is reduced expression of the prostate-specific haploinsufficient tumor suppressor protein, NKX3.1. The NKX3.1 gene is subject to loss at chromosome 8p21 and/or methylation (1). Intracellular levels of the NKX3.1 protein are reduced in prostate intraepithelial neoplasia, a noninvasive precursor to prostate cancer (1), and in regions of inflammatory atrophy that are precursors to malignant transformation in the prostate (2). Gene targeting studies in mice suggest that haploinsufficiency of Nkx3.1 is semidominant because Nkx3.1+/- mice develop prostatic dysplasia with longer latency than Nkx3.1-/-mice and loss of a single allele cooperates with Pten loss to accelerate the development and increase the severity of prostate cancer (3, 4). Additionally, prostate epithelial cells are subjected to a dose-response of Nkx3.1 protein levels because proportionately altered expression of downstream Nkx3.1 transcription targets is shown in Nkx3.1+/- mice (5). In human prostate cancer, NKX3.1 expression is reduced in primary disease (1) and completely abrogated in most metastatic foci, suggesting a continued selection for loss of the protein during prostate cancer progression (6).Control of NKX3.1 protein levels are under the influence of many factors including those that result in NH2-terminal threonine phosphorylation that results in prolongation of protein half-life (7).

Inflammation has been implicated in carcinogenesis in a number of human cancers. For example, transformation of human prostate epithelial cells occurs adjacent to foci of inflammatory atrophy. Inflammation causes the generation of reactive oxygen species that increase the risk of oxidative damage of DNA and generation of mutations (8). Inflammation of the prostate is a risk factor for the development of prostate cancer (9, 10). This report addresses the mechanism by which NKX3.1 is reduced in regions of prostatic inflammation. We have previously shown that inflammatory cytokines such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-1h accelerate NKX3.1 protein loss by inducing rapid ubiquitination and proteasomal degradation. Phosphorylation of a number of proteins is known to mediate association with ubiquitin ligase and subsequent proteasomal degradation. Computer-based analysis of the C-terminal 51 amino acids of NKX3.1 contain three potential phosphorylation sites, all proximal to amino acid 200, at serines 185, 195, and 196 (11). New data generated in 2008 that is presented in this report shows that the C-terminal domain distal to the homeodomain of NKX3.1 is not the site of ubiquitination, but is phosphorylated at specific serine residues to mediate either steady-state or cytokine-mediated protein degradation.

## Body

**Previous Data (2007):** We have previously characterized intracellular protein turnover to determine the half-life of NKX3.1 and characterized the mechanism by which protein degradation occurred. In both PC-3 and LNCaP prostate cancer cells, exogenously expressed NKX3.1 has a half-life of one hour and removal of the domain that lies c-terminal to the homeodomain increases protein half-life. We have previously shown that Bortezomib, a reversible proteasome inhibitor, prolonged half-life of endogenous and exogenous full-length NKX3.1 but had no effect on the level of exogenous C-terminal truncated protein. Additional studies showed that a MYC-tagged NKX3.1 fusion protein had a half-life similar to endogenous NKX3.1 in LNCaP cells treated with cycloheximide, indicating that both endogenous and exogenous MYC-tagged NKX3.1 were subjected to similar mechanisms of protein turnover. TNF-alpha accelerated degradation of full-length MYC-tagged protein. In contrast, truncation of the C-terminal domain prolonged protein half-life and conferred resistance to the effect of TNF-alpha on protein loss. In the presence of bortezomib, full length molecular weight moieties of NKX3.1 were seen that represented polyubiquitinated NKX3.1 accumulating in PC-3 cells, however no ubiquitination of NKX3.1(1-183) was seen under the same conditions.

**New Data (2008):** Our next goal was to determine which resides in the C-terminal domain were implicated in protein stability and to determine how the phosphorylation state of NKX3.1 affects protein turnover. To determine what region of the C-terminal domain influenced NKX3.1 stability, we engineered a series of MYC-tagged deletion constructs. The stability of these deletion proteins were tested in LNCaP cells. We have previously shown that deletion at amino acids 216, 208, or 200 has no effect on steady-state turnover of NKX3.1. Additionally, our lab has previously shown that deletion at amino acid 183 prolonged protein half-life and that deletion at 192 also prolonged protein half-life, albeit to a lesser degree. The

MYC-NKX3.1 constructs truncated at amino acids 192 and 183 were also less sensitive to the effect of bortezomib on protein turnover. In new data, we calculated protein steady state levels and half-life of these MYC-NKX3.1 constructs in response to TNF-alpha in LNCaP cells. Truncation at amino acid 192 resulted in an increased protein half-life and inhibition of the effect of TNF-alpha (Fig. 1). Truncation at amino acid 183 caused a further increase in protein half-life. Each of these serines was individually mutated to an alanine residue to abrogate each putative phosphorylation site. In addition, we made compound serine/alanine mutant constructs. The serine mutants were tested both for protein turnover and for sensitivity to TNF-alpha (Fig. 1, bottom). Mutation of serine 185 doubled the half-life of NKX3.1 and also increased protein half-life after TNF-a exposure from 25 to 40 minutes. However, NKX3.1(S185A) retained sensitivity to TNF-alpha, suggesting that serine 185 had a major influence on protein degradation but was not targeted by TNF-alpha. Mutation of either serine 195 or serine 196 prolonged protein half-life. The serine 195 mutation attenuated and the serine 196 mutation abrogated the effect of TNF-alpha on protein degradation because there was no change in the half-life of NKX3.1(S196A) after exposure to TNF-alpha. Mutation of both serines 195 and 196 enhanced the protein half-life and resistance to TNF-alpha more than the effect of the serine 196 mutant alone. The compound mutant with altered serine 185 and serine 195 showed a half-life of 110 minutes but retained an effect of TNF-alpha. In contrast to the serine 185, 196 compound mutant had a prolonged protein half-life and essentially no TNF-alpha sensitivity. Lastly, simultaneous mutation of serines 185, 195, and 196 resulted in a protein with no sensitivity to TNF-alpha and a half-life similar to the NKX3.1(1-183) construct. Thus, the effect of the C-terminal truncation on protein turnover and TNF-alpha sensitivity was regenerated by mutation of three serines 185, 195, and 196.

To demonstrate that the C-terminus of NKX3.1 was the direct target for TNF-alpha–induced phosphorylation, we performed immunodetection of phosphoserine on MYC-tagged NKX3.1 constructs. LNCaP cells were transfected with a MYC-NKX3.1 expression vector and pretreated with cycloheximide before 15 minutes of exposure to TNF-alpha. As shown in the left of Fig. 2A, exposure to TNF-alpha increased the presence of phosphoserine residues that were sensitive to treatment with CIP (Fig. 2A, right). TNF-alpha induced serine phosphorylation only on the C-terminus as shown in Fig. 2B because C-terminal truncation abolished immunodetection with anti-phosphoserine antibody. In contrast, deletion of the NH2- terminal domain upstream from the homeodomain did not affect TNF-alpha–induced serine phosphorylation. Mutation of serine 196 to alanine specifically abrogated TNF-alpha–induced serine phosphorylation. The effect was not changed by concurrent mutation of serine 195 to alanine (Fig. 2C). We were also able to show that in the presence of cycloheximide alone, mutation of serine 185 to alanine diminished detection with the anti-phosphoserine antibody, whereas mutation of serines 195 and 196 or C-terminal truncation to amino acid 192 had no effect on NKX3.1 serine phosphorylation in the presence of cycloheximide. Lastly, the C-terminal serine mutations were also found to decrease the polyubiquitination of NKX3.1 in the presence of bortezomib and in response to TNF-alpha (Fig. 3). Thus, C-terminal serines determined both ubiquitination and protein loss after exposure of LNCaP cells to TNF-alpha.

## Key Research Accomplishments

A research paper based on the work presented in the annual report for year 2007 as well as work done in 2008 was accepted to Cancer Research and published in September 2008. New data derived after the submission of the 2007 annual report and included in the cancer research paper is presented below (Figures 1-3). (Cancer Res 2008; 68: (17). September 1, 2008 6896-6901)

**Reportable Outcomes**

**1) We report the half-life of NKX3.1 steady state levels and TNF-alpha induced turnover of NKX3.1**.

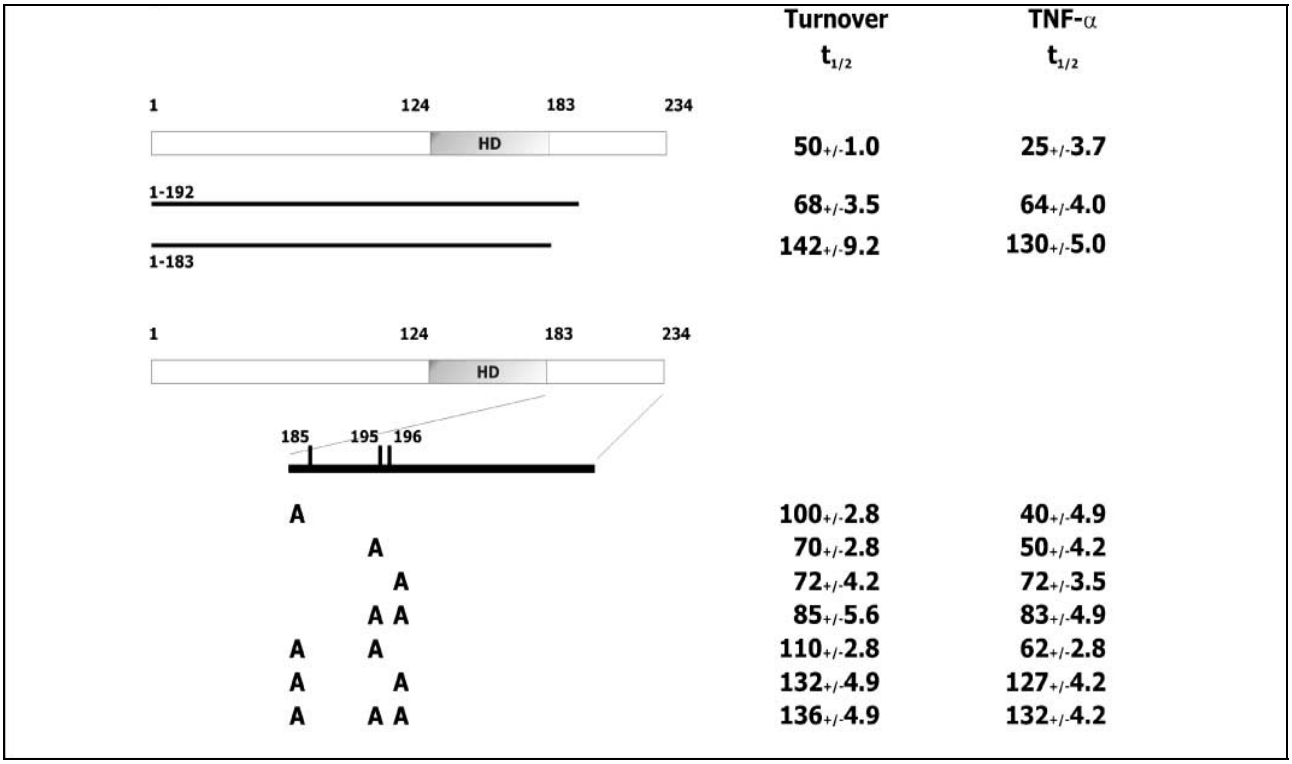


Figure 1. Determinants of NKX3.1 steady-state and TNF-a–induced turnover. The MYC-tagged NKX3.1 deletion constructs and point mutants were analyzed for half-life after 0, 30, 60, and 120 min of exposure to cycloheximide or to cycloheximide + 40 ng/mL TNF-a. NKX3.1 proteins were detected by Western blotting. Left, maps of the mutant constructs. Right, protein half-lives during turnover or after exposure to TNF-a. At least three separate determinations were done for each value. Mean standard deviations are shown for each half-life.

**2) We report the effect of TNF-alpha on NKX3.1 phosphorylation**.

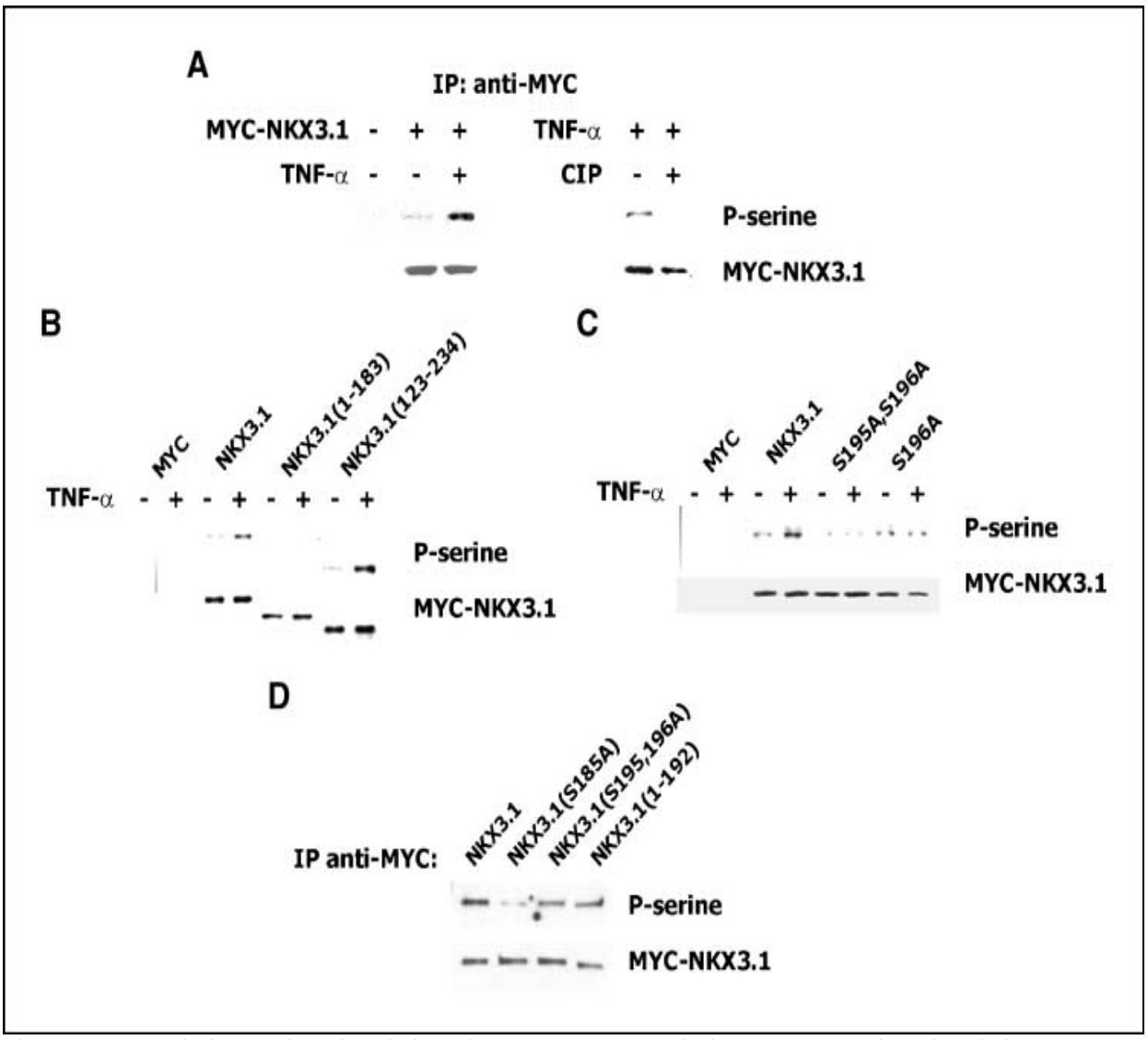


Figure 2. TNF-a induces phosphorylation of NKX3.1. A, TNF-a induces NKX3.1 phosphorylation. LNCaP cells, transfected with MYC-tagged NKX3.1, were pretreated with 100 Amol/L cycloheximide for 15 min and then exposed to 40 ng/mL TNF-a for 15 min. Right, blotting was done after one aliquot was treated with CIP. Cells were harvested for immunoprecipitation (IP) with polyclonal anti-MYC antibody followed by Western blotting with either monoclonal anti-MYC or anti-phosphoserine antibody. In B, the effect of COOH-terminal truncation and, in C, S196 and S195 mutations abolish NKX3.1 phosphorylation induced by TNF-a. D, MYC-tagged NKX3.1 constructs as indicated were expressed in LNCaP cells subjected to CHX treatment for 30 min. Immunoprecipitation was with anti-MYC antibody and immunoblotting was done with either anti-phosphoserine antibody or anti-MYC antibody.

**2) We report the effect of Ubiquitination on steady state turnover of NKX3.1 in the presence and absence of TNF-alpha**.

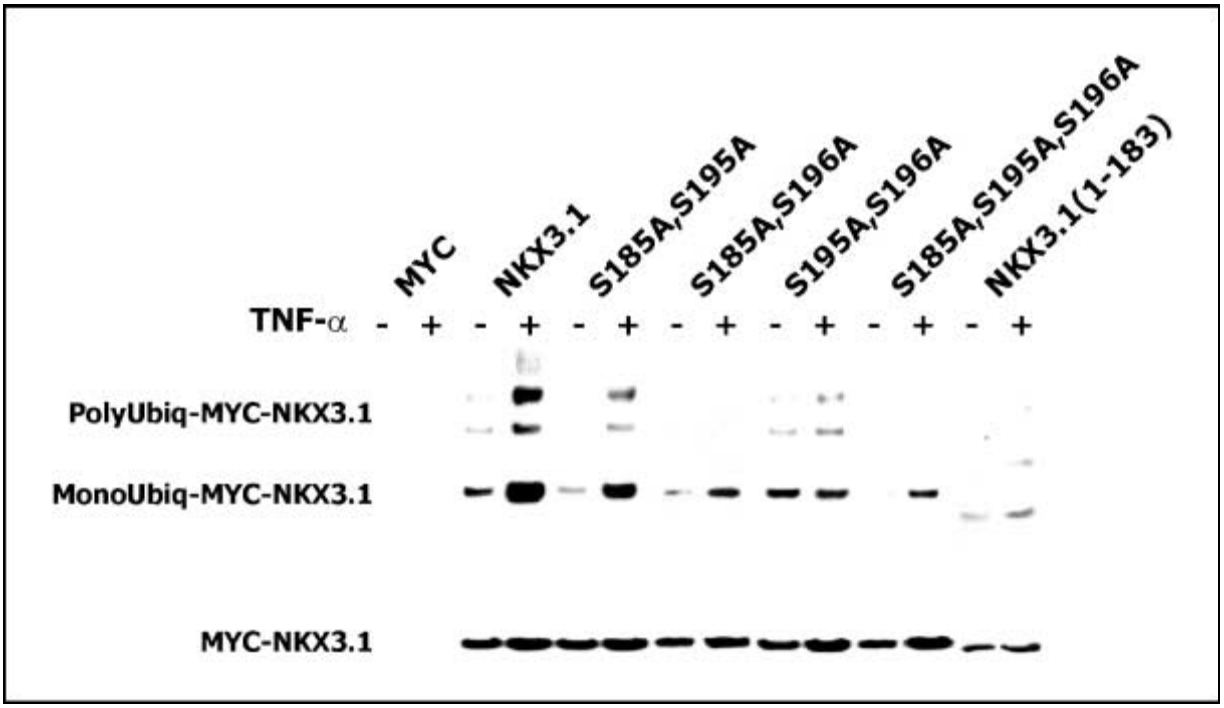


Figure 3. Determinants of NKX3.1 ubiquitination in steady-state turnover and after exposure to TNF-a. Ubiquitination of wild-type and mutant MYC-tagged NKX3.1 after cells were treated with bortezomib with or without subsequent exposure to TNF-a was analyzed in LNCaP cell extracts. An expression vector for polyhistidine-tagged ubiquitin was cotransfected into the LNCaP cells. Ubiquitinated proteins were pulled downed by Ni2+ beads and analyzed by Western blotting using an anti-MYC antibody.

## Conclusion

We conclude that C-terminal phosphorylation initiates ubiquitination and protein degradation both for purposes of NKX3.1 turnover and for degradation in response to TNF-alpha, and may extend to other inflammatory cytokines as well although this has not been tested yet. Experiments with NKX3.1 mutants showed that serine 185 was targeted for steady-state protein turnover and serine 196 was targeted by TNF-alpha. The data suggest that serine 195 had a modulating effect on the effects mediated by both serine 185 and serine 196, further augmenting both steady-state turnover and TNF-alpha–induced degradation. However, phosphorylation at serine 196 seems to be the primary signal for TNF-alpha–induced degradation of NKX3.1. Our data showed how inflammation may enhance degradation of a short-lived suppressor protein in prostate epithelial cells. These data provide a mechanism for direct and specific degradation of a suppressor protein by inflammatory cytokines. We propose that prostatic inflammation results in reduced intracellular levels of NKX3.1 and thereby predisposes cells to oncogenic transformation. We propose that prostate epithelial cell proliferation and transformation are favored by loss of NKX3.1, and therefore, selection of clones with NKX3.1 genetic loss or methylation provide an irreversible growth advantage that frequently represents the first steps in prostate carcinogenesis. In light of this, efforts to prolong NKX3.1 protein half-life are a logical strategy for prostate cancer prevention. The data suggests that anti-inflammatory agents may have activity in prostate cancer prevention. Prostate cancer prevention or treatment might be approached by inhibition of NKX3.1 ubiquitination. Because we have shown that NKX3.1 ubiquitination is mediated by phosphorylation, it is conceivable that identification of the kinase that targets the NKX3.1 C-terminal domain may be a future target for a prostate cancer prevention or treatment strategy. Moreover, the activation of TNF-alpha receptor is affected by drugs such as etanercept that interfere with ligand availability in the treatment of inflammatory diseases. Thus, there are therapeutic agents available to test the effects of TNF-alpha inhibition on NKX3.1 expression and prostate epithelial cell transformation.

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